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Role of pH in Oxidase Variability of *Aeromonas hydrophila*

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Some strains of *Aeromonas hydrophila* may be oxidase negative or only weakly oxidase positive by the Kovacs method when taken from the surface of a differential medium, such as MacConkey agar. Six strains of *A. hydrophila*, two oxidase variable, one oxidase constant, and three weakly oxidase positive on MacConkey agar, were studied to determine the cause of oxidase variability. The bacteriostatic dyes in MacConkey agar were considered possible inhibitors of the oxidase reaction. The concentration of these dyes was varied from twice the normal concentration to zero. No change in the oxidase reaction of any of the six strains was noted. Carbohydrate utilization was also studied. When lactose was deleted from the MacConkey agar formula, the oxidase-variable and weakly oxidase-positive strains became strongly oxidase positive. When glucose was substituted for lactose in the MacConkey agar formula, all strains became oxidase negative. Substitution of nonfermentable carbohydrates, such as dulcitol or raffinose, returned all strains to the oxidase-positive state. When trehalose, which is utilized by all of the strains, was substituted for lactose, the oxidase-variable strains and two of the three weakly oxidase-positive strains became oxidase negative. The other weakly oxidase-positive strain remained weakly positive and the oxidase-constant strain remained strongly oxidase positive when trehalose was substituted for lactose. Oxidase reactions were found to be negative when the pH of the medium was 5.1 or lower. Negative oxidase reactions could be reversed by raising the pH above 5.2, and positive oxidase reactions could be reversed by lowering the pH to 5.1. Therefore, the fermentation of lactose in MacConkey agar results in the inhibition of the oxidase reaction. The acid end products of the fermentation of lactose include acetic, formic, lactic, oxaloacetic, pyruvic, and succinic acids.

The oxidase test is a useful tool in differentiating groups of bacteria; it distinguishes between those bacteria that contain cytochrome *c* in their electron transport system and those that contain a different cytochrome or only slight amounts of cytochrome *c*. Bacteria containing cytochrome *c* oxidize the reagent *N,N,N,N,*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), causing the production of a blue color (1). Those bacteria which do not contain cytochrome *c* do not oxidize TMPD, and the reagent remains reduced and nearly colorless. This test has wide application for the taxonomic differentiation between the *Enterobacteriaceae* and other gram-negative rods, such as the *Pseudomonadaceae* and *Vibrionaceae*.

Aeromonas hydrophila is by definition oxidase positive, a primary characteristic used to differentiate it from other fermentative gram-negative rods (3). However, some strains of *A. hydrophila* have a false-negative oxidase reaction when the isolate is removed from gram-negative selective and differential media, such as MacConkey agar (MAC). These strains have a positive oxidase reaction when removed from a general-purpose medium, such as sheep blood agar (6). The incidence of such oxidase-variable strains is approximately 8.0% (7). It has recently been reported that low pH in the range of 4.7 to 5.6 is responsible for the negative oxidase reaction for strains of *A. hydrophila* growing on selective and differential media (4).

It has been recommended that oxidase testing be conducted only on general-purpose media (4, 6, 7). However, cultures of many clinical specimens contain multiple colonial morphologies on differential media which may not be clearly distinguished on a general-purpose medium. In such cases, if a strain of oxidase-variable *A. hydrophila* is suspected on the selective and

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differential medium, the colony must be streaked onto a general-purpose medium and incubated overnight before the oxidase activity is tested, resulting in delayed identification.

This report describes the role of low pH in suppressing the oxidase reaction of some strains of *A. hydrophila*, thus supporting the recently published work of Havelaar et al. (4), and demonstrates that, in the system described, the inhibition of the oxidase reaction occurs at a very specific pH.

MATERIALS AND METHODS

Bacterial strains. Six *A. hydrophila* strains previously characterized as oxidase variable or oxidase constant (7) were used. Strain S37 is oxidase constant because it is oxidase positive on both general-purpose and differential media. Strains S378 and UK76 are oxidase variable. Strains S32, S38, and S289 are usually weakly oxidase positive on differential media but strongly oxidase positive on general-purpose media.

Effect of inhibitory dyes. MAC was prepared, using individual nutritional ingredients (Difco Laboratories, Detroit, Mich.) rather than commercially supplied dehydrated medium to vary the dye concentration and to substitute other sugars for lactose in the formula (2). The neutral red (Sigma Chemical Co., St. Louis, Mo.) and crystal violet (Eastman Kodak Co., Rochester, N.Y.) concentrations were varied as follows: (i) both dyes at twice (2X) the normal concentration (1X); (ii) crystal violet, 2X, and neutral red, 1X; (iii) crystal violet, 1X, and neutral red, 2X; (iv) crystal violet, deleted, and neutral red, 1X; and (v) crystal violet, 1X, and neutral red, deleted. Glucose, trehalose, raffinose, dulcitol, and cellobiose were substituted for lactose at a 1.0% (wt/vol) concentration, except as noted. The medium was autoclaved at 121°C and 15 lb/in^2 for 7 min to avoid decomposition of the carbohydrates, allowed to cool to room temperature, inoculated, and incubated for 20 h in an ambient atmosphere incubator at 35°C, and the colonies were oxidase tested by the Kovacs method (5).

Effects of carbohydrates. A basal medium for the assay of the carbohydrate concentration necessary to cause inhibition of the oxidase reaction was prepared with the following: 1% (wt/vol) peptone (Difco Laboratories), 1.5% (wt/vol) agar (Difco), and 0.5% (wt/vol) sodium chloride, in distilled water. After the mixture was brought to a slow boil, glucose, lactose, or galactose was added at 1.0, 0.5, 0.25, 0.12, or 0.1% (wt/vol), and the medium was autoclaved, inoculated, and incubated; the colonies were oxidase tested as described above.

Effect of pH. To determine pH changes and their effect on oxidase activity, Mueller-Hinton broth (MHB; Difco Laboratories) was prepared in 100-ml volumes and brought to a slow boil. Carbohydrates were added at a 1.0% (wt/vol) concentration, except as noted. Each flask was thoroughly mixed on a magnetic stirrer while cooling to room temperature. The Mueller-Hinton broth-carbohydrate solutions (MHB-CHO) were each filter sterilized, and 5.0-ml aliquots were added to sterile test tubes. Random test tubes were selected from each MHB-CHO lot, and the pH was determined with a Corning Model 7 pH meter (Corning Scientific Instruments, Medfield, Mass.). Any lot of MHB-CHO that varied by more than 0.1 pH unit from pH 7.4 was discarded. MHB-CHO tubes were inoculated with the *A. hydrophila* strains to the turbidity of a McFarland no. 0.5 standard and incubated as described above. After incubation, the final pH of each culture was determined. The cultures were then transferred to conical centrifuge tubes and centrifuged in an IEC model CL centrifuge (International Equipment Co., Div. of Damon Corp., Needham Heights, Mass.) for 15 min at 1,790 × *g*. The supernatant fluid was decanted and frozen at −70°C for gas-liquid chromatographic analysis of the metabolic end products. The conical centrifuge tubes were placed at a 45° angle for 15 min to prevent the supernatant fluid from draining back onto the sedimented bacterial pellet. A portion of the bacterial pellet was removed with a platinum loop, and the oxidase activity was tested by the Kovacs method (5). To determine whether the oxidase reaction of the bacterial sediment could be reversed, those strains which had a negative oxidase reaction were grown a second time in MHB-CHO. After the initial sedimentation, the bacterial pellet was resuspended and washed in 10.0 ml of pH 7.2 phosphate-buffered 0.85% (wt/vol) saline. The suspensions were centrifuged a second time, and the drained bacterial sediments were then tested for oxidase activity.

Critical pH determination. Bacteria from 20-h cultures on sheep blood agar (5.0% [wt/vol] defibrinated sheep blood in tryptic soy agar; Difco Laboratories) were suspended in 0.85% saline at pH 7.2 to the turbidity of a McFarland no. 10 standard and adjusted to the desired pH with 25% (vol/vol) sulfuric acid. The suspensions were thoroughly mixed and allowed to stand for 5 min. After centrifugation, the supernatant fluids were decanted and the pH of each was determined. If the pH of the supernatant fluid had changed from that of the original suspension, that experiment was repeated. The drained bacterial sediments were tested for oxidase activity.

Gas-liquid chromatography. Gas chromatgraphic analyses were conducted on MHB-glucose and MHB-lactose extracts, using a Varian Aerograph model 3600 flame-ionization detector gas chromatograph and a Varian Aerograph model CDS 111 digital integrator (Varian Instruments, Palo Alto, Calif.). The chromatograph was fitted with a glass column (6 ft [ca. 183 cm] by 4 mm [inside diameter]) commercially packed with 10.0% SP-1000–1.0% H_3PO_4 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, Pa.). Operating conditions were as follows: column temperature, 150°C for volatile fatty acids and 115°C for nonvolatile fatty acids; detector block temperature, 220°C; injector temperature, 175°C; and nitrogen carrier gas flow rate, 40 ml/min. Sample size was 5 μl. Identification of peaks was accomplished by comparing retention times with those of known standards (Supelco, Inc.).

RESULTS

Alteration of the concentration of either or both bacteriostatic dyes in MAC to twice the

normal concentration or alternate deletion of each dye had no effect on oxidase activity. The oxidase-constant strain S37 and the weakly oxidase-positive strains S32, S38, and S289 remained oxidase positive, and the oxidase-variable strains S378 and UK76 remained oxidase negative.

Removal of lactose from MAC converted the oxidase-variable strains to oxidase-constant strains. The weakly oxidase-positive strains became strongly oxidase positive when lactose was deleted from MAC. Substitution of various carbohydrates for lactose in MAC caused inhibition of oxidase activity in some strains of *A. hydrophila*. Cellobiose, trehalose, and glucose, but not dulcitol or raffinose, inhibited the oxidase reactions in strains S378 and UK76, the oxidase-variable strains, and decreased the oxidase activity of strains S32, S38, and S289 (Table 1). Oxidase activity in strain S37, the oxidase-constant strain, was inhibited only by glucose.

Varying the concentration of carbohydrates in basal medium demonstrated that the strains were inhibited at different concentrations. The oxidase-constant strain S37 was oxidase negative when grown in the presence of 1.0% glucose (Table 2). Strain S378 did not regain oxidase activity until the concentration fell below 0.25%, whereas strain UK76 was oxidase negative until the concentration fell below 0.12%. The other three strains were only slightly suppressed at 1.0% when lactose was present, whereas 1.0% glucose completely inhibited the oxidase reaction.

Carbohydrate utilization by *A. hydrophila* resulted in a significant lowering in the pH of the medium (Table 3). Strains S378 and UK76 lowered the pH of the medium significantly when cellobiose, trehalose, glucose, or lactose was present but only slightly when dulcitol or raffinose was present. Strain S37 yielded low pH values when trehalose or glucose was present; however, oxidase activity was inhibited only when glucose was utilized. Strains S32, S38, and S289 did not lower the pH of the medium as much as did S378 and UK76. They were suppressed in oxidase activity only when glucose or trehalose was substituted for lactose in MAC.

The negative oxidase reactions which occurred in the presence of fermentable carbohydrates were reversed by washing the bacterial cells in pH 7.2 phosphate-buffered saline.

The oxidase reaction was positive for all strains until the environmental pH fell to 5.1, at which point the oxidase reaction became negative in all strains (Table 4).

Gas-chromatographic analysis of the 20-h MHB-glucose extracts demonstrated that *A. hydrophila* produced lactic acid in a moderate amount, succinic acid as the major product, and trace amounts of oxaloacetic and pyruvic acids in the nonvolatile fatty acids fraction, and the volatile fatty acids fraction yielded acetic and formic acid. Analysis of MHB-lactose extracts revealed the same metabolic products for all strains except S37, which does not utilize lactose and does not produce any detectable acids.

DISCUSSION

Since as many as 8.0% of *A. hydrophila* strains are oxidase negative by the Kovacs method when removed from differential media (7), it is necessary to test isolated colonies from a general-purpose medium, provided they can be differentiated from the colonies of other gram-negative rods which may be present. If the suspect colonies cannot be found on the primary plate of general-purpose medium, the colony must be

TABLE 1. *Effect of substitution of carbohydrates in MAC on the oxidase reaction of A. hydrophila*[a]

Strain	Effect with carbohydrate						
	Cellobiose	Dulcitol	Raffinose	Trehalose	Lactose	Glucose	None
Control[b]	+[c]	+	+	+	+	+	+
A. hydrophila							
S378	−[d]	+	+	−	−	−	+
UK76	−	+	+	−	−	−	+
S37	+	+	+	+	+	−	+
S289	+	+	+	−	w+[e]	−	+
S32	+	+	+	w+	+	−	+
S38	+	+	+	−	w+	−	+

[a] Oxidase reaction as determined by the Kovacs method.
[b] *Pseudomonas aeruginosa* ATCC 27853.
[c] +, Positive color change within 10 s.
[d] −, Negative or no color change within 10 s.
[e] w+, Weak positive or slight color change within 10 s.

TABLE 2. *Effects of varying the concentration of carbohydrates on the oxidase reaction of A. hydrophila*[a]

Strain	Effect at concentration														
	1.0%			0.5%			0.25%			0.12%			0.10%		
	Glu[b]	Lac[c]	Gal[d]	Glu	Lac	Gal	Glu	Lac	Gal	Glu	Lac	Gal	Glu	Lac	Gal
Control[e]	+[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A. hydrophila															
S378	–[g]	–	–	–	–	–	–	–	–	w+[h]	+	+	+	+	+
UK76	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
S37	–	+	w+	w+	+	+	w+	+	+	+	+	+	+	+	+
S289	–	w+	w+	w+	w+	+	+	+	+	+	+	+	+	+	+
S32	–	w+	w+	w+	w+	+	+	+	+	+	+	+	+	+	+
S38	–	w+	w+	w+	w+	+	+	+	+	+	+	+	+	+	+

[a] The Kovacs method used to test for oxidase activity.
[b] Glu, Glucose.
[c] Lac, Lactose.
[d] Gal, Galactose.
[e] *P. aeruginosa* ATCC 27853.
[f] +, Positive or color change within 10 s.
[g] –, Negative or no color change within 10 s.
[h] w+, Weak positive or slight color change within 10 s.

TABLE 3. *Effect of the final pH of Mueller-Hinton broth containing various carbohydrates on the oxidase reaction of A. hydrophila*

Carbohydrate[a]	Effect on *A. hydrophila* strain											
	S378		UK76		S37		S289		S32		S38	
	pH[b]	Ox[c]	pH	Ox	pH	Ox	pH	Ox	pH	Ox	pH	Ox
Cellobiose	5.1	–[d]	4.9	–	6.8	+[e]	6.7	+	6.4	+	5.8	+
Dulcitol	6.5	+	5.2	w+[f]	6.8	+	6.8	+	6.5	+	6.0	+
Raffinose	7.2	+	7.2	+	7.2	+	6.6	+	6.7	+	6.8	+
Trehalose	5.1	–	4.9	–	5.3	+	5.1	–	5.2	w+	5.1	–
Glucose	4.9	–	4.9	–	5.0	–	5.0	–	5.0	–	5.0	–
Lactose	5.0	–	4.9	–	6.7	+	5.2	w+	5.2	w+	5.2	w+
Lactose (0.10%)	6.5	+	6.4	+	6.9	+	5.7	+	6.3	+	6.5	+
None	7.3	+	7.3	+	7.1	+	7.0	+	6.8	+	6.9	+

[a] Carbohydrates were present at a concentration of 1.0%, except as noted.
[b] pH, Final pH of the culture media.
[c] Oxidase reaction by the Kovacs method.
[d] –, Negative or no color change within 10 s.
[e] +, Positive or formation of blue color within 10 s.
[f] w+, Weak positive or slight color change within 10 s.

subcultured from the differential medium. The oxidase test would be performed on colonies removed from the general-purpose medium after overnight incubation. However, this may result in delaying the final identification of the isolate. By identifying the inhibitor of the oxidase reaction in differential media, it may be possible to prevent delays and errors in the identification process.

Experiments were conducted to determine if the dyes in MAC might inhibit the oxidase reaction by donating electrons to cytochrome *c*, thus keeping it in the reduced state. Oxidized cytochrome *c* is required to react with TMPD to produce the blue color of the positive oxidase test (1). Neither doubling of the dye concentrations nor deletion of the dyes had any effect on the inhibition of the oxidase reaction of the oxidase-variable strains.

It had been previously noted that those strains of *A. hydrophila* which were oxidase variable were also lactose fermenters. However, not all of the lactose-fermenting strains of *A. hydrophila* were oxidase variable (7). These observations suggested that lactose might be responsible for the inhibition of the oxidase reaction on differ-

TABLE 4. *Effect of reducing the pH on the oxidase reaction of A. hydrophila*[a]

Strain	Effect at pH								
	5.9	5.7	5.5	5.3	5.2	5.1	5.0	4.9	4.8
Control[b]	+[c]	+	+	+	+	+	+	+	+
A. hydrophila									
S378	+	+	+	+	w+[d]	−[e]	−	−	−
UK76	+	+	+	+	w+	−	−	−	−
S37	+	+	+	+	w+	−	−	−	−
S289	+	+	+	+	w+	−	−	−	−
S32	+	+	+	+	w+	−	−	−	−
S38	+	+	+	+	w+	−	−	−	−

[a] Oxidase reaction performed by the Kovacs method.
[b] *P. aeruginosa* ATCC 27853.
[c] +, Positive or formation of blue color within 10 s.
[d] w+, Weak positive or slight color change within 10 s.
[e] −, Negative or no color change within 10 s.

ential media. This theory is supported by the fact that the oxidase-variable strains regained their oxidase activity when lactose was removed from the MAC formula. Substitution of other fermentable carbohydrates for lactose in the MAC formula resulted in inhibition of the oxidase reaction, whereas the substitution of nonfermentable carbohydrates did not. The previously reported suppression of the oxidase reaction in *A. hydrophila* on differential media (6, 7) is due to the presence of lactose and the ability of the oxidase-variable strains to ferment lactose. These results support the recent findings of Havelaar et al. (4) that the oxidase reaction of a wide variety of oxidase-positive organisms, including members of the genus *Aeromonas*, is negative between pH 4.7 and 5.6.

The critical pH for the inhibition of the oxidase reaction in strains of *A. hydrophila* appears to be 5.1. Strain S37, an oxidase-constant strain, does not ferment lactose, and the pH remains in the range of 6.7 to 6.9. The oxidase-variable strains S378 and UK76 ferment lactose and lower the pH to about 5.0.

Some strains of *A. hydrophila* produce colonies on MAC which are pink in the center and clear on the edge. These strains have weakly positive oxidase tests. The colonial appearance of these strains suggests that they do not utilize lactose as readily as do the oxidase-variable strains. This is apparently true since strains S289, S32, and S38 lower the pH to 5.2 and remain weakly oxidase positive. It has been found that strongly oxidase-positive organisms pass through a transition to weakly oxidase positive before becoming oxidase negative, when grown in the presence of a fermentable carbohydrate (4). In light of the data presented here, such a transition is probably due to the pH of the medium reaching the critical range of 5.1 to 5.2. At pH 5.2, the organisms were still oxidase positive but only weakly so. At pH 5.1, the organisms were oxidase negative.

If the concentration of a fermentable carbohydrate is reduced from 1.0% to 0.1%, strains regain their oxidase positivity as less acid is produced from the limited amount of carbohydrate present. It is also possible to reverse the negative oxidase reaction of oxidase-variable strains growing in the presence of fermentable carbohydrates by removing the bacterial cells from their acid environment and washing them in pH 7.2 phosphate-buffered saline. Reversal of negative oxidase reactions by raising the pH to 8.5 has also been reported (4).

Gas-liquid chromatography studies were performed to determine whether oxidase-constant, oxidase-variable, and weakly oxidase-positive strains produced different metabolic end products from the utilization of lactose. Analysis by gas chromatography showed that the *A. hydrophila* strains produced formic and acetic acids in the volatile fatty acid extracts of the culture medium. Pyruvic, lactic, oxaloacetic, and succinic acids were produced in the nonvolatile fatty acid extracts. There were no differences in the types or amounts of acids produced by these strains, except that the oxidase-constant strain produced no acids from lactose. This latter strain produced the same acids in the fermentation of glucose, as did the other strains. Previous gas chromatography studies of the products of *Aeromonas* fermentation yielded the same results, with the exception that oxaloacetic and pyruvic acids were not detected (8). Of these end products, succinic acid is the major component. Lowering the pH of peptone-water cultures of *A. hydrophila* by the addition of succinic acid results in converting the oxidase reaction from positive to negative (4). Similar results have been obtained using acetic, formic, and lactic acids. Therefore, the major acid end products of carbohydrate fermentation are individually or collectively able to inhibit the oxidase reaction by lowering the pH to the critical value of 5.1.

The suppression of the oxidase reaction of *A. hydrophila* growing on differential media is due to the low pH resulting from the fermentation of carbohydrates. This suppression occurred in a very narrow pH range of 5.1 to 5.2, suggesting that a very specific, but as yet unknown, mechanism may be involved.

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